

Exhibit A

Coordinate dual-gene transgenesis by lentiviral vectors carrying synthetic bidirectional promoters

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Transferring multiple genes into the same cell allows for the combination of genetic correction, marking, selection and conditional elimination of transduced cells or the reconstitution of multisubunit components and synergistic pathways. However, this cannot be reliably accomplished by current gene transfer technologies. Based on the finding that some cellular promoters intrinsically promote divergent transcription, we have developed synthetic bidirectional promoters that mediate coordinate transcription of two mRNAs in a ubiquitous or a tissue-specific manner. Lentiviral vectors incorporating the new promoters enabled efficient dual gene transfer in several tissues *in vivo* after direct delivery or transgenesis, and in a human gene therapy model. Because divergent gene pairs, likely transcribed from shared promoters, are common in the genome, the synthetic promoters that we developed may mimic a well-represented feature of transcription. Vectors incorporating these promoters should increase the power of gene function studies and expand the reach and safety of gene therapy.

Expression of multiple transgenes within the same target cells is required for several gene transfer and therapy applications¹. Gene-function studies are best performed by expressing cDNAs together with a marker gene; by this approach, genetically modified cells can be identified *in vitro* and *in vivo*. Similarly, gene therapy applications can be improved by purification of gene-corrected cells before *in vivo* administration, taking advantage of coordinate expression of selectable markers. Genetically modified cells can be amplified by introducing growth promoting or drug resistance genes together with the therapeutic gene. Conversely, genetically modified cells expressing conditionally cytotoxic genes, together with the therapeutic gene, can be eliminated *in vivo* if adverse events occur.

Coordinate expression of more than one transgene is essential when the activity to be reconstituted by gene transfer depends on multiple subunits encoded by different genes, or requires the synergism of separate molecules. In spite of such well-recognized needs, reaching coordinate, high-level expression of multiple transgenes in the majority of target cells has been a significant challenge for gene transfer technology. Two different transgenes can be expressed by two separate vectors; yet, only a fraction of target cells is transduced by both vectors

and a heterogeneous population of cells is obtained that expresses either one or two genes in different ratios. Alternatively, different promoters within the same vector have expressed two or more transgenes; yet, different tissue specificity and mutual interference between promoters often prevented efficient coexpression in the same target cells^{2,3}.

Polyproteins that self-process cotranslationally into separate components have been generated using self-cleaving 2A-like peptides of the Foot and Mouth Disease Virus (FMDV) and other picorna-viruses^{4,5}; however, application of this technology has been limited until recently because it requires molecular engineering of both transgenes and introduces sequence changes that may affect the activity, stability and immunogenicity of their protein products. The most common approach to multiple gene transfer has relied on using internal ribosome entry sites (IRES)⁶. However, several reports have noted important limitations of IRES-based dual gene transfer vectors^{7–10}, similar to those we describe here for IRES-based self-inactivating (SIN) lentiviral vectors (LVs).

To coordinately express two transgenes from a single gene transfer vector and overcome the limitations of the approaches described above, we explored a new promoter design. We joined a minimal core promoter upstream, and in opposite orientation, to an efficient promoter. The rationale of this design was that upstream elements in the efficient promoter, when closely flanked by core promoters on both sides, may drive transcriptional activity in both directions. If such bidirectional activation occurred, expression of both transcripts would be coordinately regulated.

We first tested two ubiquitously expressed promoters, previously shown to drive robust and efficient transgene expression in LVs: a 516-bp fragment from the human phosphoglycerate kinase promoter (PGK)¹¹ and a 1,226-bp fragment from the human ubiquitin C promoter (UBI C)¹². We joined them to a minimal core promoter derived from the cytomegalovirus (minCMV) that was previously developed to couple initiation of eukaryotic transcription to tetracycline-dependent operators¹³. We flanked the bidirectional promoter with two expression cassettes optimized for LV-mediated gene delivery (Fig. 1a). The upstream cassette—in antisense orientation relative to the vector LTR—included the constitutive transport element (CTE) of the Mason-Pfizer virus¹⁴, and a polyadenylation site from the Simian Virus 40 (SV40). The downstream cassette included

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Published online 26 December 2004; doi:10.1038/nbt1049

the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) and the SIN HIV-1 LTR polyadenylation site¹¹.

We cloned the luciferase reporter and the cell-associated green fluorescent protein (GFP) marker genes into the bidirectional expression cassette and generated vesicular stomatitis virus (VSV)-pseudotyped stocks of bidirectional and control monocistronic LVs. LVs carrying bidirectional expression cassettes were produced to high titer and infectivity, approaching those obtained with standard vectors. We verified correct transfer and normalized transduction of each vector stock by Southern blot analysis and/or real-time PCR of transduced HeLa cells (data not shown). We then compared gene expression in target cells transduced with equal numbers of vector copies. The

bidirectional design significantly enhanced transcription from the upstream minimal promoter without affecting downstream expression from the efficient promoter (Fig. 1b–h). Luciferase expression from the minCMV promoter, for instance, increased at least one log when fused upstream to the PGK promoter (Fig. 1b). Remarkably, the bidirectional PGK promoter allowed us to detect GFP (or a truncated version of the low-affinity NGF receptor, ALNGFR, data not shown) to the same frequency and to similar expression levels in cells transduced by the bidirectional vector and expressing the protein from either side of the promoter (Fig. 1c,d), as in cells transduced by the control PGK vector (Fig. 1e). Using two cell-associated markers, ALNGFR and GFP, we showed stable, efficient and coordinate

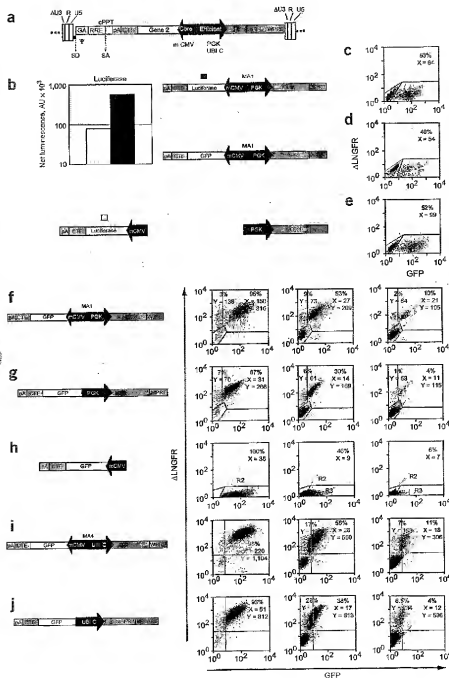


Figure 1 Gene transfer performance of bidirectional lentiviral vectors. (a) Scheme of the proviral vector form. A bidirectional promoter made by minimal core promoter elements from the human cytomegalovirus (mCMV) joined upstream, and in opposite orientation, to an efficient promoter, derived from the human phosphoglycerate kinase (PGK) or polyubiquitin UBI-C gene, was driving divergent transcription of two RNAs. CTE, constitutive transport element from the Mason-Pfizer monkey virus; pA, polyadenylation site A from the Simian Virus 40. AU3, R and U5, ITR regions with deletion in U3, SD and SA, splice donor and acceptor site; Ψ , encapsidation signal including the 5' portion of the gag gene (GA); RRE, Rev-response element; cPPT, central polypurine tract; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element. (b–e) Net luciferase activity (b) and GFP expression (c–e) in HeLa cells transduced 5–7 d before with LVs carrying the indicated bidirectional or control expression cassettes. The frequency and mean fluorescence intensity (MFI) of GFP+ cells at FACS analysis is indicated in the dot plots to the right. Luciferase activity was determined for the two marked vectors (□, ■). X, MFI. (f–j) ALNGFR and GFP expression in HeLa cells transduced 5–7 d before with serial tenfold dilutions of LVs carrying the indicated expression cassette. The frequency of ALNGFR+ (upper left region) and ALNGFR/GFP double positive (upper right region) cells, with the respective MFI of ALNGFR (Y) and GFP (X), are indicated in the FACS dot plots. Results shown are representative of at least three experiments performed with similar results.

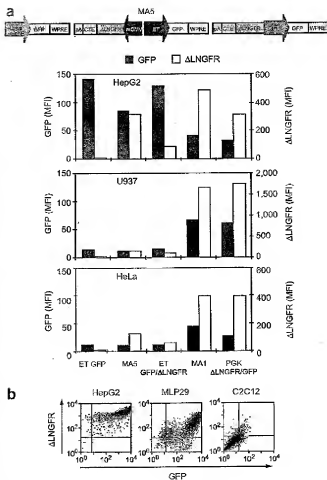


Figure 2 Tissue-specific expression by bidirectional LVs. The indicated cell lines were transduced with matched doses of the indicated monocistronic or bidirectional vectors, either based on the hepatocyte-specific ET promoter (without and with the minCMV promoter, MA5) or on the ubiquitously expressed PGK promoter (without and with the minCMV promoter, MA1; see Figure 1g and f, respectively, for schematics) and expressing GFP and ΔLNGFR, analyzed by FACS after 5 d. (a) Histograms showing the GFP (left y axis) and ΔLNGFR (right y axis) MFI in cells transduced by a low vector dose, thus representing expression from single vector copies. The mean of two determinations from one of two similar experiments are shown. (b) Representative dot plot FACS analysis of GFP and ΔLNGFR expression in target (HepG2 and MLP29) and nontarget (C2C12) cells transduced by a high dose of the hepatocyte-specific MA5 vector.

expression of bidirectional LVs, both at high and low vector copy number (Fig. 1f).

At high vector input, we reached high-level expression of both transgenes in virtually every target cell. At low vector input, when most transduced cells carried one proviral copy, we showed transgene coexpression in virtually every labeled cell, indicating the occurrence of divergent transcription from the bidirectional promoter. In both conditions, transgene expression was maintained to similar levels in cells analyzed at early and late time-points post-transduction (data not shown). Transgene-expressing cells tended to distribute along a diagonal line in the two-color fluorescence-activated cell sorting (FACS) plot, indicating that expression of the two transgenes was coordinately regulated. Moreover, by measuring expression in relation

to the number of integrated copies, as measured by real-time PCR, and analyzing cells transduced with increasing vector doses, we showed that the vast majority of integrated bidirectional vectors based on the PGK (MA1) or UBI-C (MA4) promoter efficiently expressed both transgenes (Supplementary Fig. 1 online).

Intriguingly, we observed coordinate bidirectional expression, although to substantially lower efficiency on the upstream side than the downstream side, when we tested the sole PGK promoter in the context of the bidirectional expression cassette that we developed (Fig. 1g). Bidirectional expression was detectable at single copy levels and for the vast majority of transduced cells, as assessed by real-time PCR (Supplementary Fig. 1 online). We reproduced this finding in all cell types tested (see below and data not shown) and after swapping the position of the two transgenes on each side of the PGK promoter (data not shown). These results indicated that transcription-activating elements in the PGK promoter are intrinsically capable of triggering divergent transcription and thus provide the main driving force for dual-gene expression in the new LVs, ensuring coordinate regulation of transcription on both sides of the bidirectional promoter. Apposition of the minCMV core promoter, which had a very low activity *per se* (Fig. 1b and 1h), enhanced upstream transcription from the PGK promoter possibly because of more efficient transcription initiation (compare Fig. 1f and 1g, relative GFP expression level from upstream of the PGK promoter versus upstream of the minCMV-PGK at a single vector copy: $59 \pm 4\%$, mean \pm s.d., $n = 5$, $P < 0.01$; this difference became even more apparent with increasing vector input). When we changed the driving promoter in bidirectional vectors from PGK to UBI-C, we reproduced the findings observed with the PGK promoter (Fig. 1i). We also revealed an intrinsic bidirectional activity of the UBI-C promoter (Fig. 1j) that was enhanced by the upstream addition of the minCMV promoter.

To explore the potential application of the bidirectional promoter design to tissue-specific expression, we replaced the PGK promoter in the above constructs with a hepatocyte-specific enhancer/promoter previously generated by fusing hepatocyte-specific enhancer elements to the murine transthyretin promoter (ET promoter) and tested the performance of the new LVs in a panel of cell lines of different origins transduced by matched vector doses (Fig. 2). The synthetic minCMV-ET promoter (MA5) drove efficient and coordinate expression of the upstream (ΔLNGFR) and downstream (GFP) transgenes, with both proteins highly detectable in cells transduced by a single LV copy (Fig. 2a). Notably, efficient vector expression was restricted to hepatocyte-derived (human HepG2 and murine MLP29) cells among the cell lines tested (HeLa cells, human myelocytic U937 cells, murine C2C12 myoblasts). In nontarget cells, both the original ET and the new bidirectional MA5 promoters drove a low to undetectable level of expression even after high vector input (Fig. 2b). Interestingly, as observed for the ubiquitously expressing promoters, the ET promoter displayed an intrinsic bidirectional activity in its elective target cells that was enhanced by the upstream addition of the minCMV promoter.

We then compared the dual gene transfer performance of bidirectional vectors with that observed for bicistronic vectors based either on IRES elements or self-processing peptides. For the former group we selected the encephalomyocarditis virus wild-type IRES (EMCVwt)¹⁵ as the best performing sequence among a panel of viral and eukaryotic IRESs tested (Supplementary Notes and Supplementary Fig. 2 online). For the latter group, we used the FMDV 2A peptide, whose suitability for coordinate gene transfer was recently highlighted¹⁶. We compared expression of ΔLNGFR and GFP in HeLa cells transduced with escalating doses of all these vector types by FACS, western blot

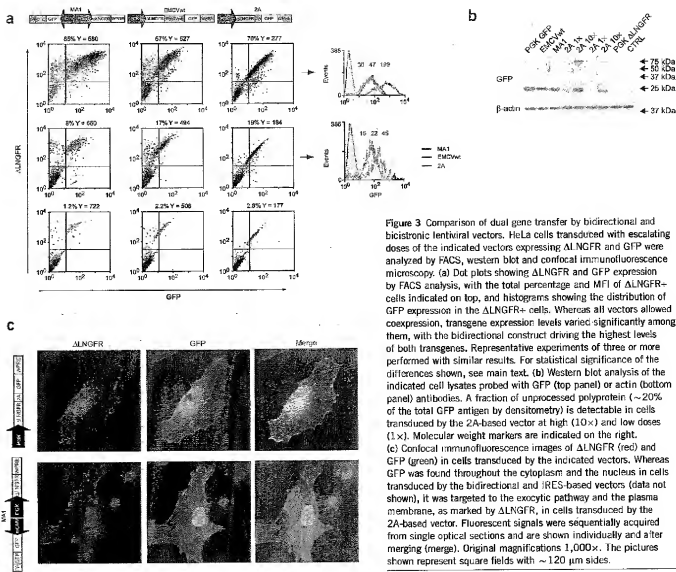


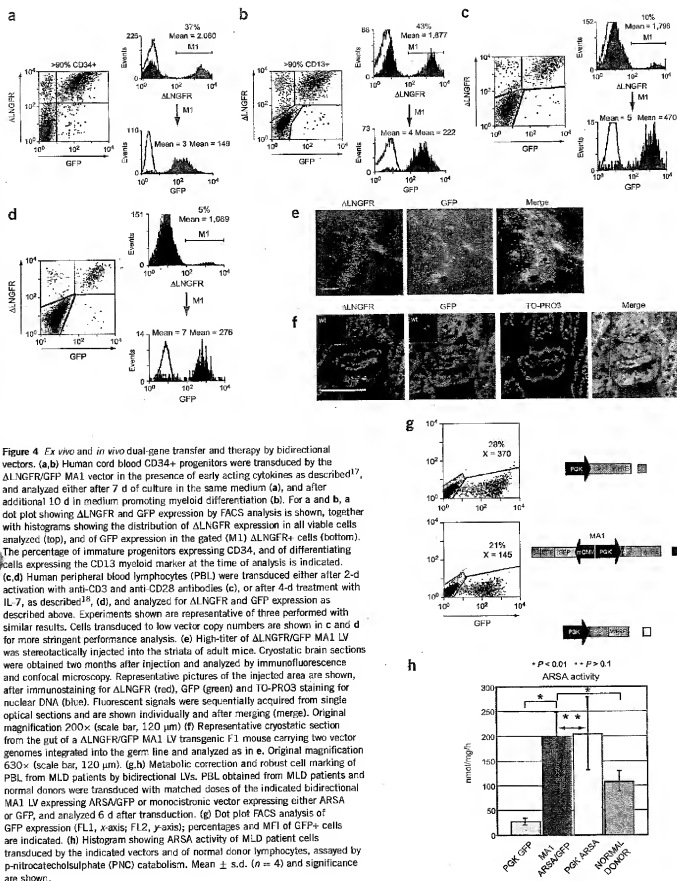
Figure 3 Comparison of dual gene transfer by bidirectional and bicistronic lentiviral vectors. HeLa cells transduced with escalating doses of the indicated vectors expressing Δ ALNGFR and GFP were analyzed by FACS, western blot and confocal immunofluorescence microscopy. (a) Dot plots showing Δ ALNGFR and GFP expression by FACS analysis, with the total percentage and MFI of Δ ALNGFR+ cells indicated on top, and histograms showing the distribution of GFP expression in the Δ ALNGFR+ cells. Whereas all vectors allowed coexpression, transgene expression levels varied significantly among them, with the bidirectional construct driving the highest levels of both transgenes. Representative experiments of three or more performed with similar results. For statistical significance of the differences shown, see main text. (b) Western blot analysis of the indicated cell lysates probed with GFP (top panel) or actin (bottom panel) antibodies. A fraction of unprocessed polyprotein (~20% of the total GFP antigen by densitometry) is detectable in cells transduced by the 2A-based vector at high ($10\times$) and low doses ($1\times$). Molecular weight markers are indicated on the right. (c) Confocal immunofluorescence images of Δ ALNGFR (red) and GFP (green) in cells transduced by the indicated vectors. Whereas GFP was found throughout the cytoplasm and the nucleus in cells transduced by the bidirectional and IRES-based vectors (data not shown), it was targeted to the exocytic pathway and the plasma membrane, as marked by Δ ALNGFR, in cells transduced by the 2A-based vector. Fluorescent signals were sequentially acquired from single optical sections and are shown individually and after merging (merge). Original magnifications $1,000\times$. The pictures shown represent square fields with $\sim 120\ \mu\text{m}$ sides.

analysis and confocal immunofluorescence microscopy (Fig. 3). Whereas all vectors enabled dual gene transfer and expression, we observed significant differences in the expression levels reached by the three types of IV. These differences were already evident when comparing cells transduced to low frequencies (between 1 and 20%), in which the vast majority of transduced cells contain only one vector copy (see also Supplementary Fig. 1 online).

Expression of Δ ALNGFR, the transgene immediately downstream to the PGK promoter in all constructs, was significantly lower for the 2A-based vector and, although to a lesser extent, for the IRES-based vector, as compared to the bidirectional vector, which expressed the protein to levels similar to those reached by monocistronic PGK vectors (average expression level $29 \pm 3\%$, $n = 4$, $P < 0.01$; and $64 \pm 17\%$, $n = 5$, $P < 0.01$, for the 2A-based and IRES-based IVs, respectively, versus bidirectional IVs). Of note, an even more significant decrease in the upstream gene expression level as compared to bidirectional or monocistronic vectors was observed from IRES-based vectors when they were used to express transgenes other than Δ ALNGFR or to transduce primary cell lines (Supplementary

Notes and Supplementary Fig. 2 online; see also the work of other groups⁸⁻¹⁰). In addition, expression of GFP from the downstream position in both bicistronic vectors was significantly lower than that observed from the upstream position in the bidirectional vector (average expression level $50 \pm 5\%$, $n = 3$, $P < 0.01$; and $40 \pm 8\%$, $n = 4$, $P < 0.01$, for the 2A-based and IRES-based IV, respectively, versus bidirectional IV). As a consequence, in cultures transduced by bicistronic vectors, only the cells expressing Δ ALNGFR to high levels reached detectable GFP coexpression.

The described differences in expression were even more apparent with increased vector input (Fig. 3a). When we monitored the self-processing of the 2A polyprotein by western blot analysis using GFP antibodies, we found that $\sim 20\%$ of the antigen in transduced cell lysates displayed a molecular weight consistent with that of an unprocessed Δ ALNGFR-2A-GFP protein (Fig. 3b). Surprisingly, expressing GFP together with the membrane-bound Δ ALNGFR in the context of a 2A peptide-based polyprotein appeared to retarget most GFP molecules, both unprocessed and processed, to the exocytic pathway (Fig. 3c).



We then assessed the performance of the bidirectional MAI LVs in more relevant targets for gene therapy applications and by different delivery strategies. We transduced human cord blood hematopoietic progenitor cells (HPCs) and adult donor peripheral blood lymphocytes (PBLs) with ALNGFR/GFP MAI LV *ex vivo*, according to previously optimized protocols^{17,18} (Fig. 4a–d). Both gene products were coordinately expressed to high levels in a large fraction of HPCs, scored both as immature cells grown in the presence of early acting cytokines (Fig. 4a) and after differentiation in liquid culture (Fig. 4b) or by clonogenic assay (data not shown). Similarly, we obtained coordinate ALNGFR and GFP expression in PBLs transduced in standard conditions of proliferation triggered by CD3/CD28 costimulation (Fig. 4c), and as nonproliferating cells, treated only with interleukin (IL)-7 to maintain naive cell properties (Fig. 4d). We also performed transplantation studies with transduced murine HPCs and showed stable coordinate expression of ALNGFR and GFP in the white blood cells of long-term engrafted mice (Supplementary Fig. 3 online). Overall, these results validated the new LVs for proficient dual gene transfer in primitive, committed and differentiated hematopoietic cells.

We then injected concentrated ALNGFR/GFP MAI LV in the striata of adult mice and scored transgene expression 4 weeks after injection by confocal microscopy of brain sections immunostained for ALNGFR and GFP (Fig. 4e). We observed robust coexpression of both transgenes in the brain tissue surrounding the injection site. We also evaluated whether the new bidirectional LVs generated dual-transgenic mouse lines¹². We obtained transgenic mice at high frequency, as assessed by the presence of vector DNA (more than 50% of newborns), and obtained vector integration in the germ line by crossing some founder mice and analyzing their progeny for vector DNA content and transgene expression. In the two F1 mice analyzed, carrying two and five vector copies in the genome, we found remarkably consistent expression of both transgenes in virtually every cell in the tissues studied, which included brain, liver, spleen, gut, heart, skeletal muscle and kidney (Fig. 4f and Supplementary Fig. 4 online). Vector expression was also easily detectable in the bone marrow and peripheral blood of the same mice, although in less than 100% of the cells, and more clearly for ALNGFR than GFP (data not shown). These data indicated that bidirectional LV transgenesis is a rapid and efficient method to obtain robust, stable and coordinate expression of two transgenes in genetically engineered mice. In addition, the data show that the minCMV-PGK bidirectional promoter that we developed governs dual transgene expression in the majority of differentiated tissues of the mouse and maintains this expression after inheritance through the germ line.

Finally, we tested the performance of the MAI bidirectional LV in a human gene therapy application using PBLs obtained from metachromatic leukodystrophy (MLD) patients, who lack functional arylsulphatase A (ARSA) in the lysosomes. We previously demonstrated that ARSA overexpression to supranormal levels in transplanted hematopoietic stem cells plays a crucial role in preventing disease manifestation in the MLD mouse model, whereas wild-type hematopoietic stem cell transplantation does not¹⁹. Here we evaluated the possibility of reproducing such conditions of overexpression in patient hematopoietic cells (Fig. 4g,h). Both bidirectional and monocistronic ARSA LVs restored enzymatic activity in patient PBLs; ARSA LVs were also overexpressed, as indicated by a two-fold increase in ARSA activity of transduced cells with respect to normal controls, at a transduction efficiency of around 20%. Moreover, using bidirectional LVs allowed genetic correction to be coupled to cell marking with the same

efficiencies as those obtained by using similar doses of monocistronic vectors expressing either ARSA or GFP.

These results demonstrate that these bidirectional LVs reach efficient dual-gene transfer and coordinate expression *ex vivo* and *in vivo* and that they outperform currently available technologies. All IRES-based LVs tested displayed some limitations because of the lower efficiency of IRES-dependent gene expression as compared to mRNA-Cap-dependent expression, and to the marked variability in IRES performance according to the type of transgene and target cell tested, as it was also reported after IRES incorporation in other types of gene transfer vectors^{6–10}. 2A peptide-based LVs were less efficient in expressing both gene products than were bidirectional vectors and expressed a fraction of unprocessed polyproteins that may display altered stability and novel antigenic features. Moreover, the cellular trafficking of the downstream fusion partner appeared to be dictated by that of the upstream partner, a surprising finding that was also reported in another recent study²⁰. The possible consequences of these expression features must be considered before adopting self-processing polyproteins for dual gene therapy.

In summary, when using bicistronic vectors, it will probably be necessary to transfer multiple vector copies or select transduced cells for efficient downstream gene expression to obtain a population expressing two transgenes in the majority of cells. Although selection protocols are compatible with some *ex vivo* gene transfer applications, they may adversely affect the biological properties of gene-corrected cells, in particular when selectable marker expression is inefficient. Even more important, the relatively low efficiency of dual gene expression by bicistronic vectors seriously limits their application to direct *in vivo* gene transfer.

By monitoring transduced cells carrying a single vector copy, we proved that divergent transcription occurred from a single bidirectional promoter, that expression of both transgenes was functionally linked and coordinately regulated, and that bidirectional promoters were consistently active in all types of target cells tested without being silenced or randomly fixed in one direction of transcription, even after cellular differentiation. Although we did not map how close the two opposite core promoters must be for operational linkage, we may expect that close juxtaposition of the fused minimal core promoter to some of the upstream elements in the efficient promoter, as observed in natural promoters between core and upstream elements, may be required. Both the PGK and UBI-C promoters tested in this work drove divergent transcription when fused to a minimal core promoter in the opposite orientation.

Intriguingly, both of these promoters were shown to be intrinsically capable of promoting divergent transcription, although to a lower efficiency on the upstream than the downstream side, when incorporated into the bidirectional expression cassette that we developed. This surprising observation may indicate a specific feature of a class of ubiquitously expressed housekeeping promoters, possibly related to their content of CpG islands (see below and refs. 21–23). Recent studies have also proposed that divergent transcription occurring from a transgenic PGK promoter may explain some unexpected experimental findings^{24,25}, providing an independent confirmation of our data in a transgenic setting.

We should also note that both promoter placement between two efficient expression cassettes endowed with post-transcriptional regulatory elements enhancing translation, and IV-mediated integration, which has been shown to preferentially target transcribed genes in the chromatin, may contribute to the unraveling of latent transcriptional activity. Although the intrinsic bidirectional activity of the housekeeping promoters tested may not be efficient enough

for exploitation *per se*, without the upstream assembly of core promoter elements described in this work, it provides the basis for the coordinate regulation of dual-gene expression obtained by our new vectors. On the other hand, the propensity of these promoters to drive divergent transcription should be kept in mind when engineering vectors and analyzing transduced cells or tissues^{24,25}, and may provide a possible mechanism for the frequently observed interference between nearby promoters in the same vector construct.

We have also extended our study to show that the bidirectional design can be successfully applied to a synthetic tissue-specific promoter to obtain coordinated dual gene expression selectively in hepatocyte cell lines. As shown for the ubiquitously expressed promoters described above, the ET enhancer/promoter displayed an intrinsic bidirectional activity that was enhanced by the upstream addition of a minCMV promoter. Further studies will clarify how frequently this feature is to be found among endogenous or synthetic promoters of different types. In addition, by combining bidirectional promoters with bicistronic transcripts one could express more than two transgenes within the same cell, although with the limitations described above.

Inducible bidirectional promoters were originally developed in Tet-regulated expression systems, by duplicating a minimal promoter on both sides of a series of Tet operator repeats to obtain exogenously regulated expression of two transgenes^{13,26}. A constitutive bidirectional promoter was recently tested for exogenous gene expression in plant biotechnology²⁷. Our results provide a description of synthetic bidirectional promoters that exploit the endogenous transcriptional machinery available to most animal cell types to drive robust and constitutive expression of two divergent transcripts.

In nature, few instances of bidirectional promoters had been documented until recently. Interestingly, a recent survey of the human genome indicated an abundance of divergently transcribed gene pairs, whose transcription start sites are separated by less than 1 kb^{28,29}. It is likely that many of the promoter elements found between these gene pairs can initiate transcription in both directions, and contain shared elements that regulate both genes³⁰. Although the functional implications of the widespread occurrence of bidirectional promoters and of antisense transcription in the human genome³¹ are not fully understood, transcription *per se* may contribute to stabilize open chromatin domains and promoter interaction with the transcriptional machinery. Thus, the synthetic bidirectional promoters that we have developed may mimic a well-represented and evolutionarily conserved feature of eukaryotic transcription, providing a structural basis for their robust performance. The new lentiviral vectors built around these bidirectional promoters will likely advance the reach and safety of gene therapy, as well as increase the power of gene-function, target validation, and animal transgenesis applications.

METHODS

Plasmid construction. All transfer vectors were built from plasmid pCCL.sin.cPPT.PGK.GFP.WPRE¹¹ using the following previously described sequence elements: EMCV IRESs with the downstream gene coding sequence starting at the 11th AIG of the IRES (wt) the MPV CTE¹⁴, a minimal CMV core promoter¹⁵, a 1,226 bp fragment from the ubiquitin C promoter¹². The ET promoter was generated by fusing a synthetic hepatocyte specific enhancer—assembled by random ligation of synthetic oligonucleotides coding for binding sites of the following hepatic transcription factors: DBP, C/EBP, HNF1, HNF3, HNF4 and HNF6, and selection for the highest expressing combination (sequence deposited in GenBank with the accession number AY661265)—to the murine transferrin promoter (M1952: 100–109 bp and M19524:128–350 bp). Detailed information on the generation and

experimental validation of the ET promoter will be reported elsewhere (A.D. Simmons, personal communication). The ALNGFR-2A-GFP polyprotein was constructed by in-frame insertion of the FMDV 2A peptide sequence between the coding sequences of ALNGFR and GFP and cloning the product into pCCL.sin.cPPT.PGK...WPRE vector. Detailed cloning procedures and sequence information are available on request.

Vector production and titration. VSV-pseudotyped third-generation LV were produced by transient four-plasmid cotransfection into 293T cells and purified by ultracentrifugation as described¹¹, with the modification that 1 µM sodium butyrate was added to the cultures for vector collection. Expression titers of GFP or ALNGFR vectors were estimated on HeLa cells by limiting dilution. Vector particle was measured by HIV-1 gag p24 antigen immunocapture (NEN Life Science Products). Vector infectivity was calculated as the ratio between titer and particle for the vector expressing GFP or ALNGFR. Vector expression titer in the 293T supernatant ranged from 0.7–1 × 10⁷ transducing units^{14,24}(TU)/ml for monocistronic CMV or PGK vector, from 3–8 × 10⁶ TU/ml for bicistronic vectors and bidirectional vectors. Vector infectivity ranged from 0.5–1 × 10⁶ TU/ml of p24 for monocistronic CMV or PGK vector, and from 2–6 × 10⁴ TU/ml of p24 for bicistronic and bidirectional vectors.

Cell cultures. Continuous cultures of HeLa and 293T cells were maintained in Iscove's modified Dulbecco's medium (IMDM; Sigma) supplemented with 10% fetal bovine serum (FBS; Gibco) and a combination of penicillin-streptomycin and glutamine. Continuous cultures of HepG2, ML219 and C2C12 cells were maintained in DMEM supplemented as above. To induce myoblast differentiation, C2C12 cultures were shifted to 2% FBS. Primary cultures of human umbilical vein endothelial cells (HUVECs), peripheral blood lymphocytes and cord blood CD34⁺ progenitors were obtained according to protocols approved by the San Raffaele Ethical Committee and maintained as described¹⁷. CD34⁺ progenitors were transduced with 5 × 10⁶ TU/ml of LV and cultured for at least 7 d in the presence of recombinant human interleukin 6 (rhIL6, 20 ng/ml), recombinant human stem cell factor (rhSCF, 100 ng/ml), recombinant human FLT-3 ligand (rhFLT-3 ligand, 100 ng/ml), all from PeproTech and recombinant human thrombopoietin (rhTPO, 20 ng/ml; Amgen) as described¹⁷. For differentiating conditions, transduced progenitors were cultured for 10 d in the presence of rhSCF, 50 ng/ml, recombinant human granulocyte-macrophage colony stimulating factor (rhGM-CSF, 20 ng/ml), recombinant human monocyte-colony stimulating factor (rhG-CSF, 20 ng/ml), all from PeproTech. For clonogenic assays, transduced cells were plated at a density of 800 cells/ml in human complete MethoCult medium (StemCell Technologies) and were scored by light and fluorescence microscopy 14 d later.

Human PBLs were purified by Ficoll gradient from healthy blood donors or MLD patients, plated in IMDM 5% human serum, stimulated either with 30 ng/ml anti-CD3 antibodies (Orthoclone) plus 1 µg/ml anti-CD28 antibodies (PharMingen) for 36–48 h, or with 5 ng/ml IL-7 (Boehringer Mannheim-Roche) for 4 d as described¹⁸, and transduced with 0.5–5 × 10⁶ TU/ml or 75–125 ng p24/ml of vector for 12 h. After transduction, PBLs were washed and grown in the presence of 6U/ml IL2.

Purification of lineage marker-negative cells from C57BL/6 mouse bone marrow with a magnetic cell depletion technique (StemCell Technologies), *ex vivo* transduction in serum-free StemSpan medium (StemCell Technologies) with 0.5–2 × 10⁶ TU/ml of vector and transplantation into lethally irradiated syngeneic recipients were done as described¹⁹.

DNA analysis Southern and real-time PCR. Vector copies per genome were quantified by real-time PCR from 300 ng template DNA extracted from cells by a commercial kit (Qiagen), using one set of primers and probe to detect the LV backbone: LV forward primer, 5'-TGAAGCGAAAGGGAAGCA-3'; LV reverse primer, 5'-CGGTGCGCGCTTCAG-3'; LV probe, 5'-(VIC)-CTCTCTGACGACGACT-(TAMRA)-3'. Reactions were carried out according to manufacturer's instructions and analyzed using the ABI Prism 7700 sequence detection system (PE-Applied Biosystems). For Southern blot analysis, DNA was extracted from transduced cells, digested with Alu-II to release the expression cassette from integrated vector DNA and analyzed with a WPRE

probe to detect vector sequences. The average number of integrated vector copies was determined relative to a plasmid standard curve.

These numbers were used to calculate vector integration rate and normalize vector stocks for all subsequent transduction experiments to ensure similar levels of integration for each vector tested.

Mice. CD1, C57BL/6⁺ and FVB mice were purchased from Charles Rivers Laboratories and maintained in specific-pathogen-free conditions. All animal procedures were performed according to protocols approved by the Hospital San Raffaele Institutional Animal Care and Use Committee.

Stereotactic injection. Nine-week-old C57BL/6 mice were anesthetized with intraperitoneal injection of tribromoethanol 1.25% (SIGMA), positioned in a stereotactic frame (David Kopf Instruments) and the skull exposed by a small incision. Two μ l of vector concentrate (2×10^6 TU/ μ l) was injected by a Hamilton syringe with a 33G blunt tip needle (Hamilton) into the left hemisphere striatum (stereotactic coordinates in mm from bregma AP = +0.74, ML = -1.9 and DV = -3.5 from skull surface) at a rate of 0.2 μ l/min. The needle was left in place for an additional 5 min before slow removal.

Transgenesis. Transgenic mice were generated using IVs as described¹². Briefly, female FVB mice were superovulated with a combination of pregnant mare serum and human chorionic gonadotropin. On average between 20 and 30 embryos were collected per female and microinjected into the perivitelline space with 10 – 100 μ l of 5×10^6 TU/ml IV stock on the same day. Manipulated embryos were immediately implanted into the oviduct of pseudopregnant CD1 mice. Pups were genotyped for the presence of the GFP sequence by PCR. Positive mice were bred to test germline transmission of the transgene. DNA was extracted from the tail and used to quantify vector copy number by real-time PCR in founder and F1 progeny mice.

Flow cytometry and luciferase assay. Transduced cells were grown for at least 4 d before FACS analysis to reach steady state GFP expression and to rule out pseudotransduction. Before FACS analysis, adherent cells were detached with 0.05% trypsin-EDTA, washed and fixed in PBS containing 1% paraformaldehyde (PAF) and 2% FBS. Cells grown in suspension were washed and resuspended in PBS containing 2 μ g/ml propidium iodide (PI) (BD Bioscience Pharmingen) and 2% FBS. For immunostaining, 10^6 cells were blocked in PBS 5% mouse serum, 5% human serum, 2% FBS for 15 min at 4 °C. After blocking, 10 μ l of R-phycoerythrin (RPE)-conjugated antibodies (anti-CD34 and anti-CD13, Dako and anti-ALNGFR, BD Bioscience Pharmingen) were added and the cells were incubated for 30 min at 4 °C, washed, stained with PI, and analyzed by three-color flow cytometry. Only viable, PI-negative cells were used for the analysis. At least 10,000 events per sample were analyzed. Luciferase was assayed in cell lysates prepared as described by the manufacturer (luciferase assay system, Promega). Relative light units were measured with a Lumat LB9507 luminometer (Berthold) after mixing cell lysates (normalized for protein content measured by a BCA Protein Assay Reagent kit from Pierce) with Luciferase Substrate (Promega).

Western blot analysis. 10^6 transduced cell were collected in Eppendorf tubes, lysed 15 min on ice in 100 μ l 50 mM TRIS, pH = 7.6, 150 mM NaCl buffer (TBS) containing 5 mM EDTA, 1% NP-40 and a cocktail of protease and phosphatase inhibitors. The lysate was cleared by centrifugation, quantified for protein content by Bradford's method, added to SDS-containing sample buffer for SDS-PAGE on 10% gel, blotted to nitrocellulose paper for 2 h at 60 V, blocked overnight in TBS–0.1% Tween–5% dry milk, washed, incubated with 1:1,000 dilution of rabbit anti-GFP antibodies (Molecular Probes) for 2 h in TBS–Tween, washed, incubated with 1:5,000 dilution of horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham), washed and developed with ECL Western Blotting detection reagent (Amersham).

Confocal microscopy analysis. Anesthetized mice were perfused with 0.9% NaCl followed by 4% PAF in PBS. Tissue samples were collected, equilibrated in 20% sucrose in PBS for 48 h at 4 °C, and embedded in optimal-cutting-temperature compound (OCT) for quick freezing. Thick cryostatic sections (10 μ m for transgenic mice and 20 μ m for stereotactically injected mice were

postfixed in PAF and frozen at -80 °C. Sections were blocked with 5% goat serum (Vector Laboratories) in PBS containing 1% bovine serum albumin (BSA) and 0.1% Triton X-100 (PBS-T), and incubated with rabbit affinity-purified GFP antibody (Molecular Probes) and R-phycoerythrin (RPE)-conjugated ALNGFR monoclonal antibody (BD Bioscience Pharmingen) for 1 h, washed and stained with Alexafluor488-conjugated goat anti-rabbit antibody (Molecular Probes) in PBS-T and 1% BSA for 1 h. Cell nuclei were stained with TOPO-3 after 1 h of RNase treatment (Molecular Probes). Sections were mounted and analyzed by three-laser confocal microscope (Radiance 2100, BioRad). Fluorescent signals from single optical sections were sequentially acquired and analyzed by Photoshop 7.0 (Adobe). For immunofluorescence analysis of *in vitro* cultures, cells were grown on poly-L-lysine-coated glass coverslips, washed, fixed in PBS containing 1% PAF and 2% FBS, permeabilized in 0.1% Triton X-100 in PBS-1% BSA for 15 min on ice, blocked and immunostained as described above.

ARSA assay. ARSA activity was determined in transduced PBL 6 d after transduction. Cells were collected and lysed in 0.5 M sodium acetate pH 5 at 4 °C for 2 h. 100 μ g of sample proteins were incubated with 100 μ l of 10 mM p-nitrocathecolate phosphate (PNC) for 90 min at 37 °C. The reaction was stopped by 1 ml of 1M NaOH and the product measured in a spectrophotometer at 515 nm. ARSA activity was expressed in nmoles PNC/mg protein/min.

Statistical analysis. Unpaired student's *t* test was performed for statistical evaluation of the data.

Note: Supplementary information is available on the Nature Biotechnology website.

ACKNOWLEDGMENTS

We are grateful to R. Loew, A.D. Simmons, D. Vignali, C. Lois and M. Samuels for providing plasmids. A. Lombardo, F. Santoni De Sio, C. Sciezko, L. Bonagante and J. Gonzalez for help with some experiments. F. Benedicenti and L. Sergi Sergi for technical help and M. De Palma for critical reading of the manuscript. This research was supported by grants from Telethon (TIGET), EU (QLK3-1999-00859 and QLRT-2001-02114), AIRC, and the Italian Ministry of Scientific Research to L.N. M.A. is a recipient of a scholarship award from O.N.A.O.S.I.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 6 October; accepted 22 October 2004

Published online at: <http://www.nature.com/naturebiotechnology/>

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Exhibit B

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